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(54) Title: VACCINES WITH ENHANCED INTRACELLULAR PROCESSING

(57) Abstract

Method for generating in a patient a cellular immune response to a target protein or portion thereof comprising the step of introducing into cells of the patient a vector containing a nucleotide sequence encoding a chimeric immunogen comprising a protein processing signal and the target protein or portion thereof, so that the chimeric immunogen is made within the cells and subsequently processed such that the target protein or portion thereof is presented to the patient's immune system so as to generate a cellular immune response.

DESCRIPTION

Vaccines with Enhanced Intracellular Processing

Field of The Invention

This invention relates to the field of immunology,
vaccination and immunotherapy.

Background of The Invention

5   The following is a discussion of the relevant art,
none of which is admitted to be prior art to the appended
claims.

DNA vaccination is a technique whereby somatic cells
are transfected *in vivo* with DNA directing synthesis of a
10 target antigen. Ulmer et al. disclose hereologous protec-
tion against influenza by injection of DNA encoding a
viral protein (Science 259:1745, 1993). Watanabe et al.
disclose the induction of antibodies to a kappa variable
region by gene immunization (J. Immunol. 151:2871, 1993).
15 The expressed protein either can be secreted by the
transfected cell or processed inside the cell and pre-
sented in the context of class I major histocompatibility
(MHC) antigens, which can be recognized by T cells. One
of the pathways whereby polypeptides are processed into
20 peptides involves intracellular proteolysis of the
polypeptide into peptide fragments that ultimately bind
MHC molecules. One major candidate process for this
pathway is that of polyubiquitination.

Ubiquitination ("Ub"), an ATP-dependent process,
25 constitutes a preliminary step of targeting a proteolytic
substrate for its eventual degradation by the proteosome,
a large multi-catalytic protease. Experiments in yeast
and rabbit reticulocyte lysates indicate that at least two
distinct determinants can dictate the rate of its
30 degradation: one is the identity of N-terminal residue (N-
end rule) and the other is presence of specific internal
lysine residue where polyubiquitin is initiated (Bachmair,

A., et al. <u>Science</u> 234:179-186, 1986; Gonda, D.K., et al. <u>J. Biol. Chem.</u> 264:16700-16712, 1989; Bachmair, A., et al. <u>Cell</u> 56:1019-1032, 1989). N-terminal amino acids are largely classified into three different categories based
5 upon their destabilizing potential and the half-life of a given protein varies significantly (from 2 min to >20h) depending on the identity of N-terminal amino acid (Bachmair, A., et al., <u>Science</u> 234:179-186, 1986; Gonda, D.K., et al. <u>J. Biol. Chem.</u> 264:16700-16712, 1989).
10 Studies have indicated that intracellular degradation of proteins is required for antigen presentation to T cells (Townsend, A., et al. <u>J. Exp. Med.</u> 168:1211-1224, 1988; Townsend, A., et al. <u>Cell</u> 42:457-67, 1985; Germain, R.N. <u>Cell</u> 76:287-299, 1994).

15   Evidence that the Ub-mediated proteolytic pathway provides all of the substrates for the proteosome has remained inconclusive from temperature-sensitive UBE1 mutant cells (Michalek, M.T., et al. <u>Nature</u> 363:552-554, 1993; Cox, J.H., et al. <u>J. Immunol.</u> 154:511-519, 1995).
20 A recent study using pairs of N-end rule substrate proteins that varied in their intracellular stability provided evidence that the proteolytic turnover of endogenously synthesized proteins is not directly proportional to the generation of processed antigenic
25 peptide/MHC class I complexes (Goth, S., et al. <u>J. Immunol.</u> 157:1894-1904, 1996). Goth et al., used the sindbis virus polymerase as the N-terminal target of the Ub-dependent degradation pathway. Earlier studies had shown that sindbis virus polymerase is a natural substrate
30 for the N-rule (de Groot et al. <u>Proc. Natl. Acad. Sci. USA</u>, 88:8967, 1991).

       Levy et al. (<u>Proc. Natl. Acad. Sci. USA</u> 93:4907, 1996) disclose a fusion protein consisting of a 21-kDa mouse DHFR moiety, an ubiquitin protein, a variable
35 residue, 165 residues of nsP4 (Sinbis virus RNA polymerase) and β gal that is useful in a method to

produce equimolar amounts of two or more specific proteins in a cell.

Grant, E.P., et al. (J. Immunol. 155:3750-3758, 1995) disclose that chimeric proteins comprising ubiquitin, a

5   destabilizing amino acid, a lacI extension and β gal when loaded into LB27.4 cells (a B lymphoblastoid cell line) showed enhanced class I presentation compared to that of proteins with a stabilizing amino acid.

Bachmair et al. U.S. Patent No. 5,496,721 disclose

10 the use of genetic constructs that encode for ubiquitin fusion proteins with destabilizing amino acids at their N-termini.

A recent study found that ovalbumin (OVA) with methylated lysine groups which was resistant to ubiquitin-

15 mediated degradation could still be presented via class I MHC, albeit at a reduced amount (Michalek, M.T., et al. J. Immunol. 157:617-624, 1996). This indicates that there may be a ubiquitin-independent pathway for class I presentation of antigens to the immune system.

20 WO 94/17816 disclose methods for the use of inhibitors of the ubiquitin-dependent proteolytic pathway to reduce cytolytic immune responses.

Summary of The Invention

The present invention concerns methods for generating

25 a cellular immune response by the introduction into cells DNA vectors encoding antigens that have enhanced rates of degradation. Such vectors allow for the production of a chimeric immunogen (protein) in the cell in which they are introduced. A chimeric immunogen includes a protein

30 processing signal and a protein which is the target for a cellular immune response. The protein processing signal brings about enhanced rates of degradation of the target protein. For example, a protein processing signal may include a removable leader linked to an intervening amino

35 acid which is linked to a ubiquitin acceptor. The protein processing signal is further linked to the target protein.

In the cytoplasm the removable leader is cleaved off by proteolytic enzymes present in the cytoplasm. This exposes at the N-terminus of the protein an intervening amino acid which acts to reduce the stability of the

5   immunogen. The chimeric immunogen contains a ubiquitin acceptor which allows for the attachment of ubiquitin by enzymes present in the cytoplasm of the cell, thus targeting the protein for degradation via the ubiquitin-proteosome pathway. Other protein processing signals that

10   utilize the intracellular proteosome pathway for degradation (with or without ubiquitin) are encompassed in the present invention.

Applicants have unexpectedly discovered that such vectors that encode for chimeric immunogens which have

15   enhanced rates of degradation via the ubiquitin-proteosome pathway are able to generate an enhanced cellular immune response. In addition, the response is limited to the cellular branch of the immune system and does not include the production of antibodies to the immunogen.

20   The present invention also concerns such vectors able to generate specific cellular immune response.

The vectors and methods of the present invention are especially useful in stimulating an immune response that can reject cancer cells or cells infected with virus.

25   This may be particularly useful in the prevention or delay of the onset of de novo or recurrent cancer or in the treatment of viral infections.

The present invention offers several advantages over prior art methods for generating an immune response. The

30   cellular immune response is greater than that achieved by the introduction of naked plasmid DNA encoding a target antigen. In addition, the ability to induce a cellular cytotoxic immune response against cells that express an antigen without inducing antigen specific antibodies

35   offers other advantages. In regard to antigens that are presented by tumor cells, the production of antibodies directed to these antigens have been hypothesized to

inhibit cellular immune responses to such antigens. Also,
such antibodies may effect the growth/survival of tumor
cells expressing an antigen that is also a signal
transducing receptor by acting as agonists of the
5   receptor.   In addition, antibodies may cause pathology
when cross reactive with self antigens.

In a first aspect the invention features a method for
generating in a patient a cellular immune response to a
target protein or portion thereof comprising the step of
10   introducing into cells of the patient a vector containing
a nucleotide sequence encoding a chimeric immunogen
comprising a protein processing signal and the target
protein or portion thereof, so that the chimeric immunogen
is made within the cells and subsequently processed such
15   that the target protein or portion thereof is presented to
the patient's immune system so as to generate a cellular
immune response.

Patients may be humans or other animals.

A cellular immune response encompasses the production
20   of cytotoxic T lymphocytes.   Cytotoxic T lymphocytes
(CTLs) are a subset of T cells that can kill target cells
expressing specific antigen(s) in the form of processed
peptides that are presented in the context of major
histocompatibility antigens (Abbas, A.K., et al. _Cellular_
25   _and Molecular Immunology_, Philadelphia: W.B. Saunders Co.,
1994b, p. 261-277).   These cells play an important role in
the immune response: (1) to intracellular infections of
non-phagocytic cells, or infections that are not
eradicated by phagocytosis, such as viral infections; (2)
30   allografts; or (3) tumors (Abbas, A.K., et al. _Cellular_
and Molecular Immunology, Philadelphia: W.B. Saunders Co.,
1994a, p. 356-375).

A target protein or portion thereof includes any
protein of interest which is subsequently degraded such
35   that peptides of the protein are presented and generate a
cellular immune response.   Tumor antigens and viral
antigens are especially preferred targets.

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There are many tumor antigens that can be recognized
by autologous CTL (Boon, T., et al. J. Exp. Med. 183:725-
729, 1996; Disis, M.L., et al. Curr. Opin. Immunol. 8:637-
642, 1996; Robbins, P.F., et al. Curr. Opin. Immunol.
5   8:628-636, 1996b). Such antigens are peptide fragments
derived from cell proteins that either are restricted to
the type of tissue from which the tumor is derived, are
mutated during the course of malignant transformation, are
aberrantly expressed by the tumor cell, and/or represent
10 "neo" antigens resulting from errors in transcription, RNA
processing, translation, and/or protein processing due to
a mutation(s) idiosyncratic to the tumor cell. Also,
viral antigens are often presented on infected cells and
on some tumor cells. There are several examples of
15 antigens that have been found to be recognized by human T
cells. These antigens include, but are not restricted to,
gp100 (Wolfel, T., et al. Eur. J. Immunol. 24:759-764,
1994; Kawakami, Y., et al. J. Immunol. 154:3961-3968,
1995), MART-1 (MelanA) (Castelli, C., et al. J. Exp. Med.
20 181:363-368, 1995), tyrosinase (Wolfel, T., et al. Science
269:1281-1284, 1995; Brichard, V.G., et al. Eur. J.
Immunol. 26:224-230, 1996; Topalian, S.L., et al. J. Exp.
Med. 183:1965-1971, 1996), MAGE-1 (Traversari, C., et al.
J. Exp. Med. 176:1453-1457, 1992; van der Bruggen, P., et
25 al. Science 254:1643-1647, 1991), MAGE-3 (Gaugler, B., et
al. J. Exp. Med. 179:921-930, 1994), BAGE (Boel, P., et
al. Immunity. 2:167-175, 1995), CAGE-1, 2 (Van den Eynde,
B., et al. J. Exp. Med. 182:689-698, 1995), N-
acetylglucosaminyltransferase-V (Guilloux, Y., et al. J.
30 Exp. Med. 183:1173-1183, 1996), (Robbins, P.F., et al. J.
Immunol. 154:5944-5950, 1995), B-catenin (Robbins, P.F.,
et al. J. Exp. Med. 183:1185-1192, 1996a), MUM-1 (Coulie,
P.G., et al. Proc. Natl. Acad. Sci. U.S.A. 92:7976-7980,
1995), CDK4 (Kawakami, Y., et al. Proc. Natl. Acad. Sci.
35 U.S.A. 91:6458-6462, 1994), Her-2 (ErbB-2)/neu (Peoples,
G.E., et al. Proc. Natl. Acad. Sci. U.S.A. 92:432-436,
1995; Fisk, B., et al. J. Exp. Med. 181:2109-2117, 1995),

human papillomavirus-E6, E7 (Ressing, M.E., et al. _Cancer Res._ 56:582-588, 1996; Alexander, M., et al. _Am. J. Obstet. Gynecol._ 175:1586-1593, 1996), and MUC-1 (Finn, O.J., et al. _Immunol. Rev._ 145:61-89. All references

5   cited herein are hereby incorporated by reference. Table 1 list the GenBank accession numbers for nucleotide sequences encoding these antigens. Utilizing known techniques of recombinant DNA technology one of ordinary skill in the art could construct chimeric immunogens which

10 contain these sequences as the target protein.

Table 1

Examples of Tumor Antigens That Can Be Modified to Enhance Their Intracellular Proteolysis[1]

Antigen	GenBank Acc.#
gp100	S73003
MART-1	U06452
TYROSINASE	M27160
MAGE-1	M77481
MAGE-2	L18920
MAGE-3	U03735
MAGE-3b	U10339
MAGE-4	D32075
MAGE-4a	U10687
MAGE-4b	U10688
MAGE-5a	U10689
MAGE-5b	U10690
MAGE-6	D32076
MAGE-8	U10693
MAGE-9	U10694
MAGE-10	U10685
MAGE-11	U10686
MAGE-41	D32077
MAGE-Xp	X82539
BAGE	U19180
N-acetylglucosaminyltransferase-V Intron	X91652
p15	U19796
MUM-1	U20896
MUM-1b	U20897
MUM-1c	US0908
ErbB-2 (HER-2/neu)	X03363
CDK4	U37022
Human papillomavirus	X64084
Human papillomavirus-E6	U34107
Human papillomavirus-E7	U76411
Prostate Specific Antigen (PSA)	M27274

[1] All sequences included in this chart are hereby incorporated by reference.

Introduction into cells of a patient can be carried
out either *in vitro* or *in vivo*. *In vitro* introduction
entails the removal of cells from a patient and subsequent
reintroduction of these cells into a patient once a vector
5   has been introduced into the cells. Techniques for the
isolation and reintroduction of cells are well known to
those who practice the art. The vector can be introduced
into the cells by standard DNA transfection techniques or
electroporation or via liposomes (Potter, H., et al. Proc.
10   Natl. Acad. Sci. USA 81:7161-715, 1984; Felgner, P.L., et
al. Nature 337:387-388, 1989; Mannino, R.J., et al.
Biotechniques. 6:682-690, 1988). Introduction of the
vector *in vivo* can be carried out by direct injection of
the vector into cells of the patient (Plautz, G.E., et al.
15   Proc. Natl. Acad. Sci. USA 90:4645-4649, 1993; Wolff,
J.A., et al. Science 247:1465-1468, 1990; Wu, G.Y., et al.
Biotherapy. 3:87-95, 1991; Herweijer, Hans, et al. Somatic
Gene Therapy, CRC Press, Inc., 1996, p. 183-202; Raz, E.,
et al. Proc. Natl. Acad. Sci. USA 91:9519-9523, 1994).
20   Preferably, cells are of skeletal muscle origin, however
other cell types are suitable for injection.

A protein processing signal is responsible for
enhancing the rate of degradation of the target protein in
the cytoplasm via the proteosome pathway. A preferred
25   protein processing signal consists of a removable leader,
an intervening amino acid, and an ubiquitin acceptor
linked together.

The removable leader is a protein sequence which is
cleaved, cotranslationally or following translation at the
30   junction of the leader and any protein sequence to which
it is attached. Cleavage is carried out by processing
proteases which are specific for the leader and which are
present in the cell in which the protein is expressed.
The leader allows for the protein to remain in the
35   cytoplasm prior to and subsequent to cleavage. Any
sequence which can be specifically cleaved in the
cytoplasm at a particular point within the expressed

protein; for example, at the junction site with the adjoining intervening amino acid is useful in the present invention. Removable leaders which are useful in the present invention include ubiquitin, which is cleaved by
5 ubiquitin specific processing proteases (Waxman, L., et al. _J. Biol. Chem._ 262:2451-2457, 1987; Orlowski, M. _Biochemistry_ 29:10289-10297, 1990) and amyloid beta protein which is cleaved by secretase (Selkoe, D.J., et al. _Ann. N.Y. Acad. Sci._ 777:57-64, 1996).

10   The intervening amino acid present is preferably positioned at the N-terminus of the protein by the cleavage of the leader sequence. The intervening amino acid when present at the N-terminus of the chimeric immunogen destabilizes the protein and thus enhances its
15 rate of degradation via the N-end rule. A preferred intervening amino acid is arginine. Preferably the rate of degradation is within minutes. Other suitable amino acid are described in Gonda, D.K., et al. _(J. Biol. Chem._ 264:16700-16712, 1989). The present invention also
20 contemplates variation in the placement of the intervening amino acid, so long as the resulting protein is rapidly degraded within the target cell.

    An ubiquitin acceptor is a molecule which contains a residue appropriately positioned from the N-terminal of
25 the protein as to be able to be bound by ubiquitin molecules. Such residues preferentially have an epsilon amino group such as lysine. Physical analysis demonstrates that multiple lysine residues function as ubiquitin acceptor sites (King, R.W., et al. _Mol. Biol._
30 _Cell_ 7:1343-1357, 1996b; King, R.W., et al. _Science_ 274:1652-1659, 1996a). Examples of other ubiquitin acceptors include lacI or Sindis virus RNA polymerase. Ubiquitination at the N-terminal of the protein specifically targets the protein for degradation via the
35 ubiquitin-proteosome pathway.

    Other protein processing signals that destabilize the target proteins and allow for enhanced intracellular

degradation via the proteosome pathway are contemplated in
the present invention. These other methods to destabilize
target proteins do not necessarily go through the
ubiquitin pathway, but all are degraded in the cytoplasm
5   via proteosomes.

The present invention contemplates the use of other
protein processing signals which govern the rate(s) of
intracellular protein degradation including, but not
limited to, those described by Bohley, P., et al. (_Biol._
10 _Chem. Hoppe. Seyler_ 377:425-435, 1996). Such processing
signals include those that allow for phosphorylation of
the target protein (Yaglom, J.A., et al. _Mol. Cell Biol._
16:3679-3684, 1996; Yaglom, J., et al. _Mol. Cell Biol._
15:731-741, 1995). Also contemplated by the present
15 invention are modification of the chimeric immunogens that
allow for post-translational arginylation (Ferber, S., et
al. _Nature_ 326:808-811, 1987; Bohley, P., et al. _Biomed._
Biochim. Acta 50:343-346, 1991) of the protein which can
enhance its rate(s) of intracellular degradation. The
20 present invention also contemplates the use of certain
structural features of proteins that can influence higher
rates of intracellular protein turn-over, including
protein surface hydrophobicity, clusters of hydrophobic
residues within the protein (Sadis, S., et al. _Mol. Cell_
25 _Biol._   15:4086-4094,   1995),   certain   hydrophobic
pentapeptide motifs at the protein's carboxy-terminus (C-
terminus) (e.g. ARINV, as found on the C-terminus of
ornithine decarboxylase (Ghoda, L., et al. _Mol. Cell Biol._
12:2178-2185, 1992; Li, X., et al. _Mol. Cell Biol._ 14:87-
30 92, 1994), or AANDENYALAA, as found in C-terminal tags of
aberrant polypeptides (Keiler, K.C., et al. _Science_
271:990-993, 1996) or PEST regions (regions rich in
proline (P), glutamic acid (E), serine (S), and threonine
(T) (Rogers, S.W., et al. _J. Biol. Chem._ 263:19833-19842,
35 1988)). Moreover, certain motifs have been identified in
proteins that appear necessary and possibly sufficient for
achieving rapid intracellular degradation. Such motifs

include RxALGxIxN region (where x = any amino acid) in cyclins (Glotzer, M., et al. Nature 349:132-138, 1991) and the KTKRNYSARD motif in isocitrate lyase (Ordiz, I., et al. FEBS Lett. 385:43-46, 1996).

5   The present invention also contemplates enhanced cellular degradation of the chimeric immunogen which may occur by the incorporation into the target protein known protease cleavage sites. For example amyloid beta-protein can be cleaved by beta- and gamma-secretase (Iizuka, T., et al. Biochem. Biophys. Res. Commun. 218:238-242, 1996) and the two-chain vitamin K-dependent coagulation factor X can be cleaved by calcium-dependent endoprotease(s) in liver (Wallin, R., et al. Thromb. Res. 73:395-403, 1994).

      The constructs of the present invention encode a target polypeptide linked to or containing a protein processing signal sequence, containing one or a combination of the aforementioned motifs and/or the required structural features, that can enhance the intracellular degradation of the polypeptide. Those of ordinary skill in the art can readily link or incorporate such protein processing signals into target proteins utilizing known techniques of recombinant DNA technology.

      In preferred embodiments the target protein is greater than 25 amino acid residues; the protein is selected from the group consisting of tumor antigens or viral antigens; the vector further comprises a mammalian promoter; the cellular immune response is the predominate immune response in the patient.

      A mammalian promoter is any promoter that will allow for transcription to be initiated in a mammalian cell. Examples of such promoters include the CMV (Cytomegalovirus), SV40 (Simian virus 40) and RSV (Rous Sarcoma Virus).

      In a second aspect, the invention features a vector comprising a nucleotide sequence encoding a mammalian promoter and a chimeric immunogen comprising a protein processing signal and a target protein or portion thereof.

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Vectors in addition to plasmids are included in the scope of the present invention such as replication-defective viral vectors including adenovirus vectors and retroviral vectors (Wu, G.Y., et al. _Biotherapy._ 3:87-95, 1991; Kipps, T.J. _J. Hematotherapy_ 2:367-372, 1993).

It is also possible to attach genetic adjuvants that can enhance the ability of the host immune system to recognize cells expressing the proteolyzed polypeptide so as to enhance the efficacy of such vaccines. For example by co-injection of genes encoding interleukin-2 (IL-2) (Raz, E., et al. _Proc. Natl. Acad. Sci. USA_ 90:4523-4527, 1993).

Brief Description of the Figures

Fig. 1 shows the construct of plasmid pcDNA3 Ub-X-lacI-lacZ.

Fig. 2 shows the detection by immunoblot of β gal expression from P815 transfectants and _in vitro_ translated protein mixture. Lane 1 is control pcDNA3, lanes 2, 4, 6 are cell lysate from P815/Ub-X-lacZ (X = Arg, Met or Tyr) transfectants and lanes 3, 5 and 7 are _in vitro_ translated protein mixture from plasmids Ub-X-lacZ. The arrow indicates β gal protein migrating at 116 kDa.

Fig. 3 shows the detection of β gal activity in four P815 transfectants by β gal assay and FACS analysis. The x and y axes represent the logarithm of the fluorescence intensity and the cell number on arbitrary scales, respectively.

Fig. 4 shows the effects of inhibitors on β gal expression in P815 transfectants. Four different P815 transfectants (shown on the x-axis) were pre-incubated with control, proteosome inhibitors LLnL and lactacystin, lysosome inhibitor chloroquine or calpain inhibitor E64-D for 2 hours at 37°C. β gal activity is shown on the y-axis.

Fig. 5 shows effects of Ub-X-β gal protein degradation on antigen presentation. E:T ratio is on the x-axis. On the y-axis is shown % specific lysis.

Fig. 6 shows detection of anti-β gal antibodies from mice immunized with different plasmids. Time after DNA injection (weeks) is shown on the x-axis. OD_{405} is shown on the y-axis.

Fig. 7 shows cellular immune responses to β gal in BALB/c mice immunized with various plasmids. E:T ratio is on the x-axis. On the y-axis is shown % specific lysis.

Figs. 8(A-F) show a diagramatic representations of a chimeric genes encoding different protein processing signals affixed to ErbB-2/Neu (Peoples, G.E., et al. *Proc. Natl. Acad. Sci. U.S.A.* 92:432-436, 1995; Fisk, B. et al. *J. Exp. Med.* 181:2109-2117, 1995).

Description of the Preferred Embodiments
Chemicals

E64-D (2S.3S-t-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester) and chloroquine were purchased from Sigma (St. Louis, MO). LLnL (N-acetyl-L-leucinyl-L-leucinal-L-norleucinal), a proteosome inhibitor (Rock, K.L., et al. *Cell* 78:761-771, 1994), was purchased from Boehringer Mannheim (Indianapolis, IN). Lactacystin is a streptomyces metabolite (purchased from Dr. Cohen, Harvard University). E64-D was dissolved in DMSO, LLnL in ethanol and the final concentration of each reagent in cell culture was kept at 0.25%. Both chloroquine and lactacystin were dissolved in water.

Cell lines

P815, a mastocytoma cell line from DBA/2 mice ($H-2^d$), was maintained in RP10 media (RPMI-1640, 10% fetal calf serum (FCS), 50 uM 2-ME, antibiotics and L-glutamine). P13.2 is P815 transfected with *Escherichia coli* β gal that was maintained in RP10 with 0.4 mg/ml G418. 0805B, a H-$2L^d$-restricted β gal-specific CTL clone, was provided by

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Dr. Michael Bevan (University of Washington, Seattle, WA) and was maintained by weekly stimulation of 5×10^4 CTL with 10^5 irradiated P13.2 and 3×10^6 irradiated splenocytes from BALB/c ($H-2^d$) mice (Jackson Laboratories, Bar Harbor, ME)
5 in 2 ml RP10, containing 50 uM 2-mercaptoethanol and 50 u/ml IL2 from supernatant of P3-IL2 transfectant cells in 24-well tissue culture plates.

Plasmids & Stable Transfectants

        Plasmid, pUB23 (Bachmair, A., et al., Science
10 234:179-186, 1986) encodes Ub-X-lacI-lacZ, a fusion protein comprised of yeast ubiquitin, X residue (X=Arg, Met or Tyr), Escherichia coli lacI segment (residues 1030-1149) and β-galactosidase (β gal). Upon expression, cleavage occurs after the last residue of ubiquitin by
15 cytosolic ubiquitin protease exposing the X-residue as the N-terminal residue. The internal lysine residue is provided in the lacI segment to serve as the ubiquitin acceptor. The chimeric genes Ub-X-lacZ were subcloned into pcDNA3, a mammalian expression vector under the
20 control of the human CMV promoter(Invitrogen, CA)to generate pcDNA3Ub-X-lacI-lacZ (X = Arg, Tyr or Met) (See Figure 1) (These plasmids are designated Ub-X-lacZ where X = Arg, Tyr, or Met). pRcCMVlacZ (lacZ) is the name of the plasmid encoding the wildtype β gal. The 5' non-
25 coding region of ubiquitin was modified by introducing a Kozak sequence (GCCACC) to direct efficient translation of the chimeric gene transcripts in mammalian cells. The plasmids were transfected into P815 by electroporation. After clonal selection, the stable transfectants were used
30 for the described experiments.

β gal·Assay

        After P815 transfectants were washed in FACS buffer (RPMI/3% FCS/0.05% sodium azide), they were resuspended in 0.1 ml of same buffer and incubated for 10 min at 37°C.
35 Cells were then loaded with 0.1 ml prewarmed β gal

substrate, fluorescein di-B-D-galactopyranoside (FDG, 2 mM in water) (Molecular Probes, OR) for 1 min. at 37°C by hypotonic shock. The reaction was stopped by addition of 2 ml of ice-cold FACS buffer. Since nonfluorescent FDG is
5 hydrolyzed by β gal to flurorescine monogalactoside (FMG) and then to highly fluorescent fluorescein, intracellular β gal activity can be measured by Flow Cytometric Analysis (FACS).

Preparation of Plasmid DNA
10 DNA was prepared using Qiagen megaprep kits (Qiagen, Chatsworth, CA), with the modification of adding one-tenth volume 10% Triton X-114 (Sigma) to the filtered bacterial lysate for 30 min on ice before applying it to the column. Purified DNA was suspended in sterile saline and endotoxin
15 level was tested using a limulus extract clot assay (Associates of Cape Cod, Wood Hole, MA).

Immunization of Mice
Six to eight-week-old BALB/c mice were injected intramuscularly in the rear quadriceps with 100 ug of
20 either Ub-X-lacZ, lacZ, or the pcDNA3 as control vector in a total volume of 100 ul saline using a 25-gauge needle. Injections were given weekly for four times.

Antibody Assays
Anti-β gal antibodies were measured by ELISA.
25 Microtiter plates were coated overnight with 5 ug of β gal (Calbiochem, La Jolla, CA) per ml of phosphate-buffered saline (PBS, pH 7.4) and then washed with PBS. Nonspecific binding sites were then blocked with 1% bovine serum albumin in PBS. After washing four times in
30 PBS/0.5% Tween 20, serum samples diluted 1:100 in PBS were added to the wells. After 1 hr incubation at room temperature (RT), the plates were washed with PBS/Tween 20 and incubated with alkaline phosphatase-labeled goat anti-mouse IgG (Pharmingen, CA) for 1 hr at RT. The plates were

then washed with PBS/Tween 20 and p-nitrophenyl phosphate (5 mg/ml, Sigma), an alkaline phosphatase substrate was added. The level of anti-β gal Ab was determined by absorbance at 405 nm read 30 min after addition of the substrate. Results are expressed in OD.

Cytotoxicity Assay

Splenocytes from immunized mice were isolated 12 weeks after injection. 7×10^6 responder splenocytes were incubated with 0.5×10^6 stimulator P13.2 which was irradiated at 20,000 rads in RP10. 5 days later, 25 U/ml recombinant murine IL-2 (Biosource International, CA) was added to the culture and incubated for another 2 days. Then the restimulated cells were harvested and separated from dead cells on a Lymphocyte M (Accurate Chemicals, Westbury, NY) gradient. The targets were P815 and P13.2, as negative and positive control, respectively. In 96-well round-bottom plates, target cells were incubated with responder cells at different effector to target ratio for 4 h. in phenol red-free RPMI-1640 containing 2% BSA, 2 mM glutamine and 1% penicillin and streptomycin. 50 ul/well of the supernatant was then transferred to a 96-well plates and lysis was determined by measuring lactate dehydrogenase (LDH) release using the Cytotox 96 assay kit (Promega Corp., Madison, WI). The released LDH converts the added substrate tetrazolin salt (INT) into red formazan product and the amount of color is proportional to the number of lysed cells. The absorbance values from supernatant is recorded at O.D. 490 nm (O.D.) on an ELISA reader. Percent lysis is calculated as follows:

$$\frac{O.D._{Exp} - O.D._{Spon. E} - O.D._{Spon. T}}{O.D._{Max. T} - O.D._{Spon. T}} \times 100$$

$O.D._{Exp}$ = O.D. of the supernatant containing the effector cells (e.g., CTL) and target cells (e.g., tumor cells).

O.D.$_{Spon. E}$ = O.D. of the supernatant containing only effector cells.

O.D. $_{Spon. T}$ = O.D. of the supernatant containing only target cells.

5   O.D.$_{Max. T}$ = O.D. of the supernatant containing target cells that were lysed.

Administration

The phrase "suitable for human use" and "pharma-ceutically acceptable" (physiologically tolerable) refer
10   to molecular entities and compositions that do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

The term "unit dose" as it pertains to the inocula of
15   the present invention refers to physically discrete units suitable as unitary dosages for animals, each unit containing a predetermined quantity of active material calculated to produce the desired immunogenic effect in association with the required diluent; i.e., carrier, or
20   vehicle. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are directly dependent on (a) the unique characteristics of the active material and the particular immunologic effect to be achieved, and (b) the limitations inherent in the
25   art of compounding such active material for immunologic use in animals and human subjects, as disclosed in detail herein, these being features of the present invention.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be
30   therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to generate a cellular immune response, and degree of protection desired. Precise amounts of active ingredient required to
35   be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable

dosage ranges are of the order of about one hundred micrograms to about one hundred milligrams, preferably about one to about 10 milligrams and more preferably about 5 milligrams active ingredient per kilogram bodyweight

5   individual. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in one or two week intervals by a subsequent injection or other administration.

10   *Ex vivo* methods are contemplated wherein the cells into which the chimeric immunogen gene is to be introduced are isolated from the animal or patient and then the gene is introduced into those isolated cells using suitable methods. Examples of useful *ex vivo* methods have been

15   described for example by Raper, S. E., et al. Ann. Surg., 223:116, 1996; Lu, L., R. N. Shen, and H. E. Broxmeyer, Crit. Rev. Oncol. Hematol., 22:61, 1996; Koc, O.N., et al. Semin. Oncol., 23:46 1996; Fisher, L.J., et al. Curr. Opin. Neurobiol., 4:735, 1994; and Goldspiel, B. R., et

20   al. Clin. Pharm., 12:488, 1993. Following the introduction of the gene, including any optional steps to assure that the chimeric immunogen gene has been successfully introduced into those isolated cells, the isolated cells are introduced into the patient either at

25   a specific site or directly into the circulation of the patient. In preferred embodiments of the present invention, cell surface molecules, such as antigens that identify specific cells, are used to specifically isolate such desired cells from the patient. One of ordinary

30   skill in the art will understand that such isolation methods are well known and include such methodologies as fluorescent activated cell sorting (FACS), immunoselection involving a variety of formats including panning, columns, or other similar methods.

35   The present invention also contemplates introducing the chimeric immunogen gene into the desired cells within the body of an animal or human patient without first

removing those cells from the patient.   Methods for
introducing genes into specific cells *in vivo*, or within
the patient's body are well known and include use of gene
therapy vectors and direct injection of various genetic
5   constructs into the animal or patient. Examples of useful
methods have been described by Danko, I., et al. (*Vaccine*,
12:1499, 1994; Raz, E., et al. *Proc. Natl. Acad. Sci. U.
S. A.*, 90:4523, 1993; Davis, H. L., et al. *Hum. Gene
Ther.*, 4:151, 1993; Sugaya, S., et a. *Hum. Gene Ther.*,
10   7:223, 1996; Prentice, H., et al. *J. Mol. Cell Cardiol.*,
28:133, 1996; Soubrane, C., *Eur. J. Cancer*, 32A:691, 1996;
Kass-Eisler, A., *Ann. N.Y. Acad. Sci.*, 772:232, 1995;
DeMatteo, R.P., et al. *Ann. Surg.*, 222:229, 1995; Addison,
C. L., et al. *Proc. Natl. Acad. Sci. U.S.A.*, 92:8522,
15   1995; Hengge, U.R., et al. *J. Clin. Invest.*, 97:2911,
1996; Felgner, P.L., et al. *Ann. N. Y. Acad. Sci.*,
772:126, 1995; and Furth, P.A., et al. *Hybridoma*, 14:149,
1995).   In a typical application, a gene therapy vector
containing a chimeric immunogen gene is introduced into
20   the circulation or at a localized site of the patient to
allow the gene therapy vector to specifically infect the
desired cells.   The present invention also contemplates
the direct injection of DNA from a genetic construct into
a patient or animal. Examples of such useful methods have
25   been described by Vile, R. G., et al. (*Ann. Oncol.*, 5
Suppl 4:59, 1994). The genetic construct DNA is directly
injected into the muscle or other sites of the animal or
patient.

Genetic constructs

30   The chimeric immunogens of the present invention may
be constructed using standard genetic engineering methods
to operatively link a protein processing signal nucleotide
sequence to a nucleotide sequence encoding a target
protein.   In addition, standard genetic engineering
35   methods may be used to insert man-made nucleotide
sequences or sub-domain nucleotide sequences into the

target protein. One of ordinary skill in the art will
understand that various methods may be utilized to produce
such chimeric immunogens. For example, a gene conversion
method known as "SOEN" may be used to produce a chimeric
5   immunogen. The methods for using this gene conversion
method are well known in the art and have been described
for example in Horton, R.M. Mol. Biotechnol., 3:93, 1995;
Ali, S.A., et al. Biotechniques, 18:746, 1995; Vilardaga,
J.P., et al. Biotechniques, 18:604, 1995; Majumder, K., et
10 al. PCR. Methods Appl., 4:212, 1995; Boles, E., et al.
Curr. Genet. 28:197, 1995; Vallejo, A.N., et al. PCR.
Methods Appl., 4:S123, 1994; Henkel, T., et al. Anal.
Biochem., 214:351, 1993; Tessier, D.C., et al.
Biotechniques, 15:498, 1993; Morrison, H.G., et al.
15 Biotechniques, 14:454, 1993; Cadwell, R.C., et al. PCR.
Methods Appl., 2:28, 1992; and Stappert, J., et al.
Nucleic Acids Res., 20:624, 1992. Alternatively, one of
ordinary skill in the art will understand that site-
directed mutagenesis may be used to introduce changes into
20 a particular nucleotide sequence to directly produce or
indirectly be used to produce a chimeric immunogen of the
present invention. For example, the mutagen kit provided
by BioRad Laboratories may be used together with the
methods and protocols described within that kit to produce
25 the desired changes in the nucleotide sequence. These
methods were originally described by Kunkel (Proc. Natl.
Acad. Sci. USA, 82:488-492, 1985) and Kunkel et al.,
(Meth. Enzol. Mol., 154:367-382, 1987). By using the site
directed mutagenesis protocols described herein and known
30 within the art, a skilled investigator may induce
individual nucleotide changes which result in an altered
amino acid sequence or which preserve an amino acid
· sequence but introduce a desired restriction enzyme
recognition sequence into a protein processing or target
35 protein sequence. This new restriction endonuclease
recognition site may then be used to cut any sequence at
that particular point and to attach or insert another

sequence of interest. In addition to these methods, one of ordinary skill in the art will understand that an entire chimeric immunogen molecules may be synthesized using synthetic methods known in the art. This
5 methodology only requires that the skilled artesian generating nucleotide sequence of a chimeric immunogen molecule and provide that sequence to a company which is capable of synthesizing such a gene.

Promoters
10   Other promoters that are particularly useful for expressing genes and proteins within eukaryotic cells include mammalian cell promoter sequences and enhancer sequences such as those derived from polyoma virus, adenovirus, simian virus 40 (SV40), and the human
15 cytomegalovirus. Particularly useful are the viral early and late promoters which are typically found adjacent to the viral origin of replication in viruses such as the SV40. Examples of various promoters which have been used in expression vectors have been described by Okiama and
20 Berg (Mol. Cell. Biol. 3:280, 1983), the pMLSVN SV40 described by Kossman et al. (Nature 312:768, 1984). One of ordinary skill in the art will understand that the selection of a particular useful promoter depends on the exact cell and the other various parameters of the genetic
25 construct to be used to express the chimeric immunogen or the chimeric immunogen gene within a particular cell. In addition, one of ordinary skill in the art will select a promoter which is known to express genes in the target cell at a sufficiently high level to be useful in the
30 present invention.
      The genetic vectors and expression vectors of the present invention optionally contain various additional regulatory sequences including ribosome binding sites which allow the efficient translation of the messenger RNA
35 produced from an expression vector into proteins.

The genetic constructs contemplated by the present invention therefore include various forms of accessory genes which are operatively linked to either a promoter sequence or a promoter and enhancer sequence and also
5   operatively linked to a polyadenylation sequence which directs the termination and polyadenylation of messenger RNA. It is also contemplated that the genetic constructs of the present invention will contain other genetic sequences which allow for the efficient replication and
10 expression of that construct within the desired cells. Such sequences may include introns which are derived from native target protein genes or, for example, from a virus gene.

The present invention also contemplates gene therapy
15 vectors which are able to directly infect mammalian cells so as to introduce the desired chimeric immunogen gene into that cell. These gene therapy vectors are useful for directly infecting cells which have been isolated from an animal or patient, or can be directly introduced into an
20 animal or patient and thereby directly infect the desired cell within that animal or patient.

Many types of gene therapy vectors which are able to successfully transfer genes and cause the expression of desired foreign DNA sequences have been developed and
25 described in the literature. For example, the article entitled "Gene Transfer Vectors for Mammalian Cells" in Current Comm. Mol. Biol., Cold Springs Harbor Laboratory, New York (1987). Further, naked DNA can be physically introduced into eukaryotic cells including human cells by
30 transfection using any number of techniques including calcium phosphase transfection (Berman et al., Proc. Natl. Acad. Sci. USA, 81:7176, 1984), DEAE-Dextran Transfection, protoplast fusion (Deans et al., Proc. Natl. Acad. Sci. USA, 81:1292, 1984), electroporation, liposome fusion,
35 polybrene transfection and direct gene transfer by laser micropuncture of the cell membrane. In addition, one of ordinary skill in the art will understand that any

technique which is able to successfully express the chimeric immunogen in a cell would be useful in the present invention.

Specifically, gene therapy vectors which utilize
5   recombinant infectious virus particles for gene delivery have been widely described. See, for example, Brody, S. L., et al. Ann. N. Y. Acad. Sci., 716:90, 1994; Srivastava, A. Blood. Cells, 20:531, 1994; Jolly, D. Cancer Gene Ther., 1:51, 1994; Russell, S.J., Eur. J.
10   Cancer, 30A:1165, 1994; Yee, J.K., et al. Methods Cell Biol., 43 Pt A:99, 1994; Boris-Lawrie, K.A. et al. Curr. Opin. Genet. Dev., 3:102, 1993; Tolstoshev, P., Annu. Rev. Pharmacol. Toxicol., 33:573, 1993; and, Carter, B.J. Curr. Opin. Biotechnol., 3:533, 1992). The present invention
15   contemplates the use of gene therapy vectors to carry out the desired methodology of the present invention by introducing a gene encoding a chimeric immunogen into the cell. Many viral vectors have been defined and used as gene therapy vectors and include virus vectors derived
20   from simian virus 40 (SV40), adenoviruses, adeno-associated viruses, and retroviruses. One of ordinary skill in the art will understand that useful gene therapy vectors are vectors which are able to directly introduce into the target cells the DNA which encodes the chimeric
25   immunogen and allow that DNA to persist in the cell so as to express the chimeric immunogen in the desired manner within the cell.

The gene therapy vectors of the present invention are useful for introducing chimeric immunogen genes into a
30   variety of mammalian cells including human cells. The particular cells infected by the gene therapy vector will depend on the various specifics of the vector.

A large variety of methods are contemplated in which the final result is that the chimeric immunogen gene is
35   introduced into the desired cells. These methods include ex vivo methods, in vivo methods and various other methods

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which involve injection of DNA, genetic vectors or gene
therapy vectors into the animal or human.

        The following Examples are provided for further
illustrating various aspects and embodiments of the
5   present invention and are in no way intended to be
limiting in scope.

Example 1: β gal protein expression and β gal activity in
p815 Ub-X-lacZ transfectants

        To test Ub-X-β gal protein expression in mammalian
10 cells, three different plasmids Ub-X-lacZ (X = Arg, Met,
or Tyr) were transfected into P815 (a murine mastoocytoma
cell line) by electroporation with pcDNA3 as a negative
control. After G418 and clonal selection, cell lysates
were analyzed by SDS-PAGE-immunoblot with anti-β gal
15 monoclonal Ab and detected by Enhanced Chemiluminescense
(ECL) (Amersham, IL). As shown in figure 2 (lanes 1, 2,
4 & 6), β gal protein of 116 kDa was only detected in Ub-
Met-lacZ P815 transfectant. The β gal activity in these
transfectants also was assayed by FACS. Cells were loaded
20 with the β gal substrate, FDG, and analyzed for
fluorescence intensity by flow cytometry as previously
described. Figure 3 shows the FACS profiles obtained with
the stable transfectants. Consistent with the immunoblot,
92% Ub-Met-lacZ transfectant cells have β gal activity
25 whereas Ub-Arg-lacZ and Ub-Tyr-lacZ are the same as
negative control, pcDNA3.

        Since neither β gal protein nor its activity can be
detected in Ub-Arg-lacZ and Ub-Tyr-lacZ transfectants, the
competence of these two constructs was determined by in
30 vitro transcription and translation. An approximately 116
kDa band (same size of β gal) was detected in all Ub-X-
· lacZ constructs (Figure 2, lanes 3, 5, & 7). Furthermore,
RNA transcripts of lacZ from these transfectants also were
detected by RT-PCR (data not shown).

35   These data indicate that the proteins Ub-Arg-β gal
and Ub-Tyr-β gal may be metabolized . rapidly in the

transfected P815 cell, consistent with these proteins
being proteolyzed by a ubiquitin proteosome-dependent
pathway. Consequently, the rate of β gal protein
degradation appears much faster than that of its synthesis

5 in P815 Ub-Arg or Tyr-lacZ transfectants. Therefore, β-
galactosidase activity in these transfectants cannot be
detected.

Example 2: Effect of proteosome inhibitors on the expres-
sion of Ub-X-β gal

10   Proteosome inhibitors have been shown to inhibit
major peptidase activity of 20S and 26S proteosome
function in cells and reduce the degradation of protein
and ubiquitinated protein substrate. The effect of
different kinds of cell-penetrating proteosome inhibitors

15 on the level of β gal activity in Ub-X-lacZ transfectants
was examined. Peptide aldehyde N-acetyl-L-leucinyl-L-
leucinal-L-norleucinal (LLnL) (Rock, K.L., et al. Cell
78:761-771, 1994) is an substrate-related inhibitor of the
chymotryptic site on the proteosome. Lactacystin (Mori,

20 S., et al. J. Biol. Chem. 270:29447-29452, 1995) is a
streptomyces metabolite specific proteosome inhibitor.

        After different P815 transfectants were incubated
with proteosome inhibitors at 100 uM for 2 h at 37°C, the
cells were washed and β gal activity was determined by β

25 gal assay and FACS. LLnL caused a tremendous increase of
β gal activity in both Ub-Arg-lacZ and Ub-Tyr-lacZ
transfectants, with 81% and 44% of cells positive for
lacZ, respectively (Figure 4). Lactacystin had a stronger
effect on β gal activity than LLnL. These data indicate

30 that ubiquitin-proteosome is involved in the degradation
process of Ub-(Arg, Tyr)-β gal. Both LLnL and lactacystin
had little effect on the β gal level in the Ub-Met-lacZ
transfectant, where Met is a stabilizing residue in yeast
and rabbit reticulocyte (Bachmair, A., et al. Science

35 234:179-186, 1986; Gonda, D.K., et al. J. Biol. Chem.
264:16700-16712, 1989). Two other protein proteolysis

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pathway inhibitors also were used to confirm the involvement of proteosome in the degradation of Ub-Arg-β gal and Ub-Tyr-β gal. Treatment of cells with E64-D (50 μM), a specific calpain inhibitor or chloroquine (100 μM),

5   a lysosomal degradation inhibitor, had no effect on the level of β gal activity, further suggesting that ubiquitin-proteosome dependent proteolysis, but no other pathway is involved in the rapid degradation of the N-end rule substrates Ub-Arg-β gal and Ub-Tyr-β gal.

10 Example 3: Effects of Ub-X-β gal protein degradation on antigen presentation

An important function of intracellular proteolysis is to generate the small peptides that are bound to MHC class I molecules, transported to plasma membrane and presented

15 to cytotoxic CD8 T lymphocytes to initiate immune response. During this process, proteosomes may play a role in MHC class I presentation (Goldberg, A.L., et al. Nature 357:375-379, 1992). The fact that proteosome inhibitors can increase the level of β gal activity in

20 P815 transfectants suggests that Ub-X-β gal is degraded by ubiquitin-proteosome dependent proteolysis. Therefore the effects of increased Ub-X-β gal degradation on its antigenicity was assessed through CTL assay.

Three different P815 Ub-X-lacZ transfectants were

25 incubated with 0805B, a H-2-L^d-restricted β gal specific CTL clone for 4 h at different E:T ratios. 0805B is effector. The target is the P815 Ub-X-lacZ transfectants. The ability of the P815 transfectants to generate peptides that are appropriately presented by MHC class I molecules

30 was determined by measuring their ability to be lysed by 0805B. As shown in figure 5, all three Ub-X-lacZ transfectants are sensitive to specific cytolysis to a similar degree as positive control, P13.2, a lacZ transfectant of P815. The fact that transfectants expressing

35 β gal with destabilizing residues Arg and Tyr did not show any detectable β gal protein expression and activity but

were presented at a high degree to a CTL clone, indicate
that ubiquitin-proteosome-mediated proteolysis is an
important pathway of protein degradation leading to MHC
class I antigen presentation.

5   Example 4:   The effect of increased Ub-X-lacZ degradation
on functional immunity in vivo
The effect of engineering proteins so as to achieve
rapid degradation via the ubiquitin processing pathway on
antibody response and cytotoxic activity was examined.
10   Mice were immunized with Ub-X-lacZ (X = Arg or Met), lacZ,
or pcDNA3. Four BALB/c mice per group were injected
intramuscularly with 100 ug of plasmid DNA each week for
four weeks. The animals were bled prior to the first
injection and then each week starting 3 weeks thereafter.
15   The levels of anti-β gal Ab were detected by ELISA and
shown as O.D. + Standard Deviation (Error Bar). Only the
mice injected with lacZ have significant Ab production.
As shown in Figure 6, anti-β gal Ab was detected by three
weeks after the first injection from mice injected with
20   lacZ.   The levels of anti-β gal continuously increased in
such mice for another 3 weeks. Injection of Ub-Arg-lacZ
did not induce production of detectable anti-β gal Ab.
One mouse injected with Ub-Met-lacZ stimulated antibody
production significantly (data not shown). This may
25   reflect the differences in the intracellular stability of
protein and the need for a larger protein to produce an
antibody response.
To determine if Ub-X-lacZ gene could induce a
specific CTL response, mice were sacrificed 12 weeks after
30   injection. Splenocytes were restimulated in the presence
of P13.2, a lacZ transfectant of p815 that presents $H-2^d$-
restricted CTL epitopes of the β gal protein. The spleen
cells were assayed 7 days later for their ability to lyse
the lacZ-expressing target cell-line, P13.2, or P815, the
35   lacZ negative parental cell line.   Ub-Arg-lacZ induced

much stronger specific CTL than both Ub-Met-lacZ or lacZ
(Figure 7).

This indicates that the Ub-Arg-lacZ construct is not
only able to specifically generate a cellular immune
5   response, but that this immune response is significantly
greater than that induced by lacZ constructs with greater
intracellular stability.

Example 5:   Her-2

Over-expression of proto-oncogenes can lead to neo-
10   plastic transformation. The *neu* oncogene originally was
identified by its ability to transform NIH 3T3 cells *in
vitro* (Padhy, L.C., et al. Cell 28:865-871, 1982).
Subsequently, *neu* was found to be highly homologous to a
gene on human chromosome 17 (17q21), designated *erbB-2
15   (HER-2/ neu)* (Schechter, A.L., et al. Science 229:976-978,
1985), which is a cell surface growth factor receptor.
ErbB-2 is over-expressed in 15-40% of all human breast
cancers (Slamon, D.J., et al. Science 235:177-182, 1987;
van de Vijver, M.J., et al. N. Engl. J. Med. 319:1239-
20   1245, 1988; Kraus, M.H., et al. EMBO J. 6:605:619, 1987;
King, C.R., et al. Cancer Res. 49:4185-4191, 1989). This
association may define a causal relationship as indicated
by studies on mice transgenic for the activated or wild-
type *neu* proto-oncogene under the control of the mouse
25   mammary tumor virus (MMTV) promoter. Transgenic mice
expressing activated-*neu* develop multiple mammary tumors
at an early age (Muller, W.J., et al. Cell 54:105-115,
1988;   Bouchard, L., et al. Cell 57:931-936, 1989).
Moreover, transgenic mice with the wild-type *neu* gene
30   under the MMTV promoter also develop focal mammary tumors,
albeit with slower kinetics (Guy, C.T., et al. Proc. Natl.
·Acad.· Sci USA 89:10578-10582, 1992).   The relative
selectivity of erbB-2 overexpression in human adeno-
carcinomas and the association of erbB-2 and *neu* with a
35   pathogenic mechanism responsible for neoplasia, make the
protein product of these genes an attractive target for

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immunotherapy (Fendly, B.M., et al. _J. Biol. Response Mod._ 9:449-455, 1990; Fendly, B.M., et al. _Vaccine Res._ 2:129-139, 1993).

Depicted in Figure 8 are constructs of the proto-oncogene product of ErbB-2/neu that are engineered to have enhanced rates of intracellular proteolysis. One or a combination of the various motifs may be used to optimize the ability of genes encoding the chimeric protein to induce a cellular immune response when injected into somatic cells of the animal. Construct (A) (Fig. 8A) has the ubiquitin (Ub) encoded by the 5' end of the gene. X is for the desired intervening amino acid (e.g. Arg) that will become the amino terminus after removal of the ubiquitin moiety. This construct relies on internal ubiquitin acceptor site(s) within the target antigen (e.g. in this case erbB-2/neu) for subsequent poly-ubiquination. Construct (B) (Fig. 8B) has an ubiquitin acceptor sequence of the lacI region interposed between the Ub-X and the target antigen (e.g. erbB-2/neu). Construct (C) (Fig. 8C) encodes the target antigen with an altered carboxy-terminus containing one or more "AANDENYALAA" motifs. Construct (D) (Fig. 8D) encodes the target antigen with an altered carboxy terminus containing a "ARIVN" motif. Construct (E) encodes the target antigen with an altered carboxy terminus containing two or more "ARINV" motifs. Construct (F) (Fig. 8F) encodes the target antigen with an altered carboxy terminus containing one or more "PEST" domains and one or more "ARINV" motifs.

These constructs are useful for the generation of a CTL response specific to the neu protein expressed on tumor cells. Immunotherapy would entail injection of such constructs into patients having tumor cells over-expressing neu on their cell surface.

Other embodiments are within the claims.

-30

Claims

        1.   Method for generating in a patient a cellular
immune response to a target protein or portion thereof
comprising the step of:
  5   introducing into cells of said patient a vector
containing a nucleotide sequence encoding a chimeric
immunogen comprising a protein processing signal and said
target protein or portion thereof, so that said chimeric
immunogen is made within said cells and subsequently
 10   processed such that said target protein or portion thereof
is presented to said patient's immune system so as to
generate a cellular immune response.

        2.   The method of claim 1, wherein said protein
comprises greater than 25 amino acid residues.

 15   3.   The method of claim 1, wherein said protein or
portion thereof is selected from the group consisting of
tumor antigens and viral antigens.

        4.   The vector of claim 1, wherein said protein
processing signal comprises a removable leader, an
 20   intervening amino acid, and an ubiquitin acceptor.

        5.   The vector of claim 4, further comprising a
mammalian promoter, and wherein said removable leader is
a ubiquitin molecule, and said intervening amino acid is
arginine.

 25   6.   The method of claim 1, wherein said cellular
immune response is the predominant immune response in said
patient.

        7.   A vector comprising a nucleotide sequence
encoding a mammalian promoter and a chimeric immunogen
 30   comprising a protein processing signal and a target
protein or portion thereof.
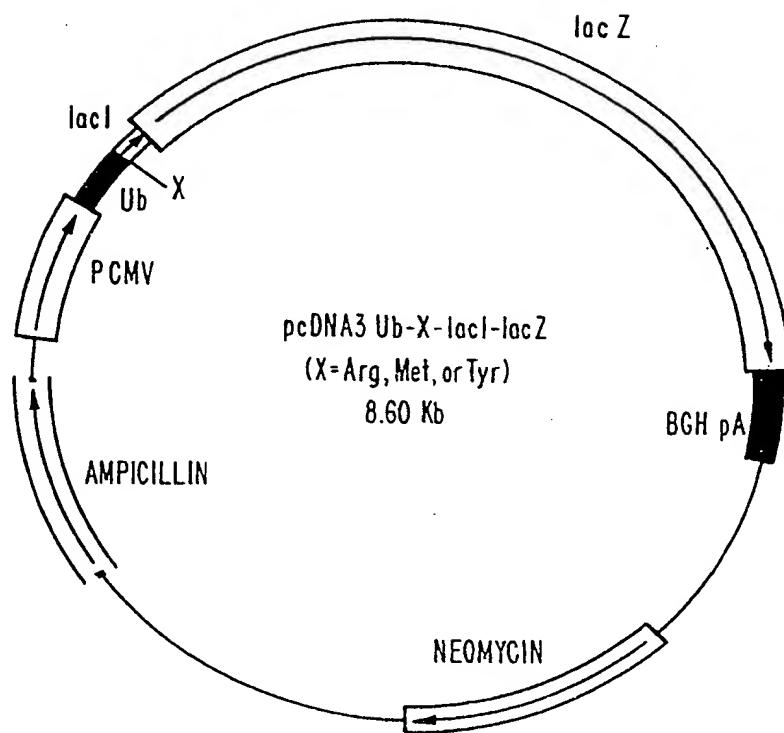
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FIG. 1.

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FIG. 2.

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FIG. 3

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FIG. 4.
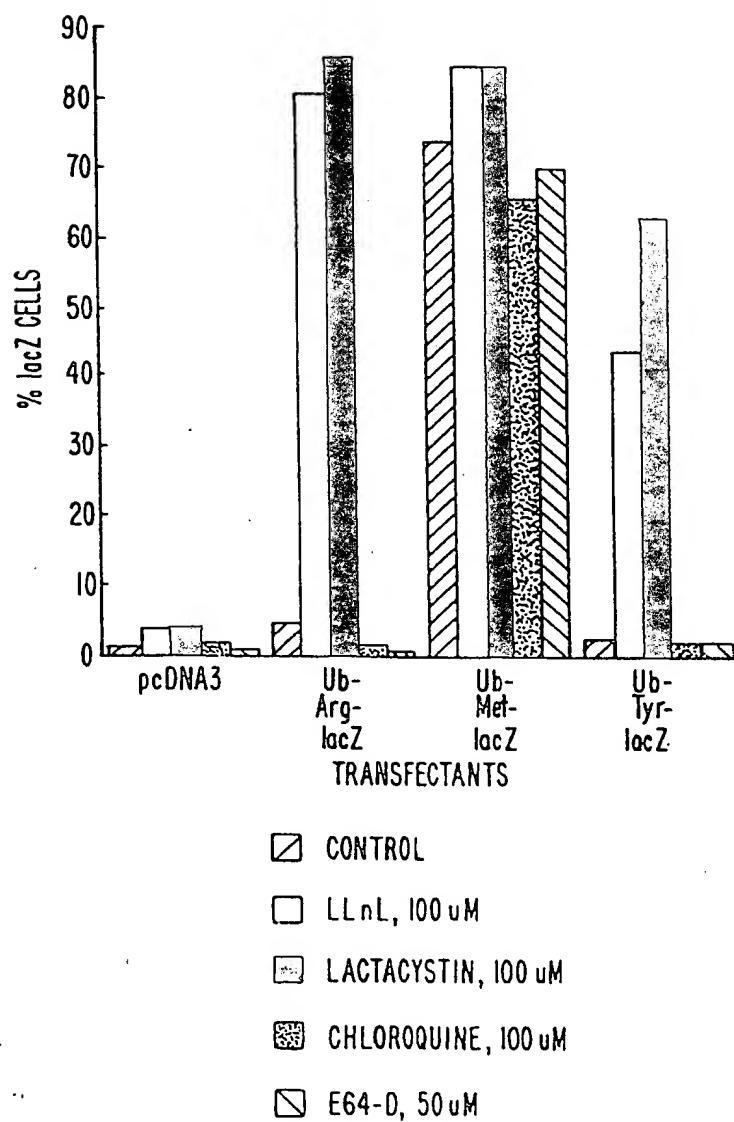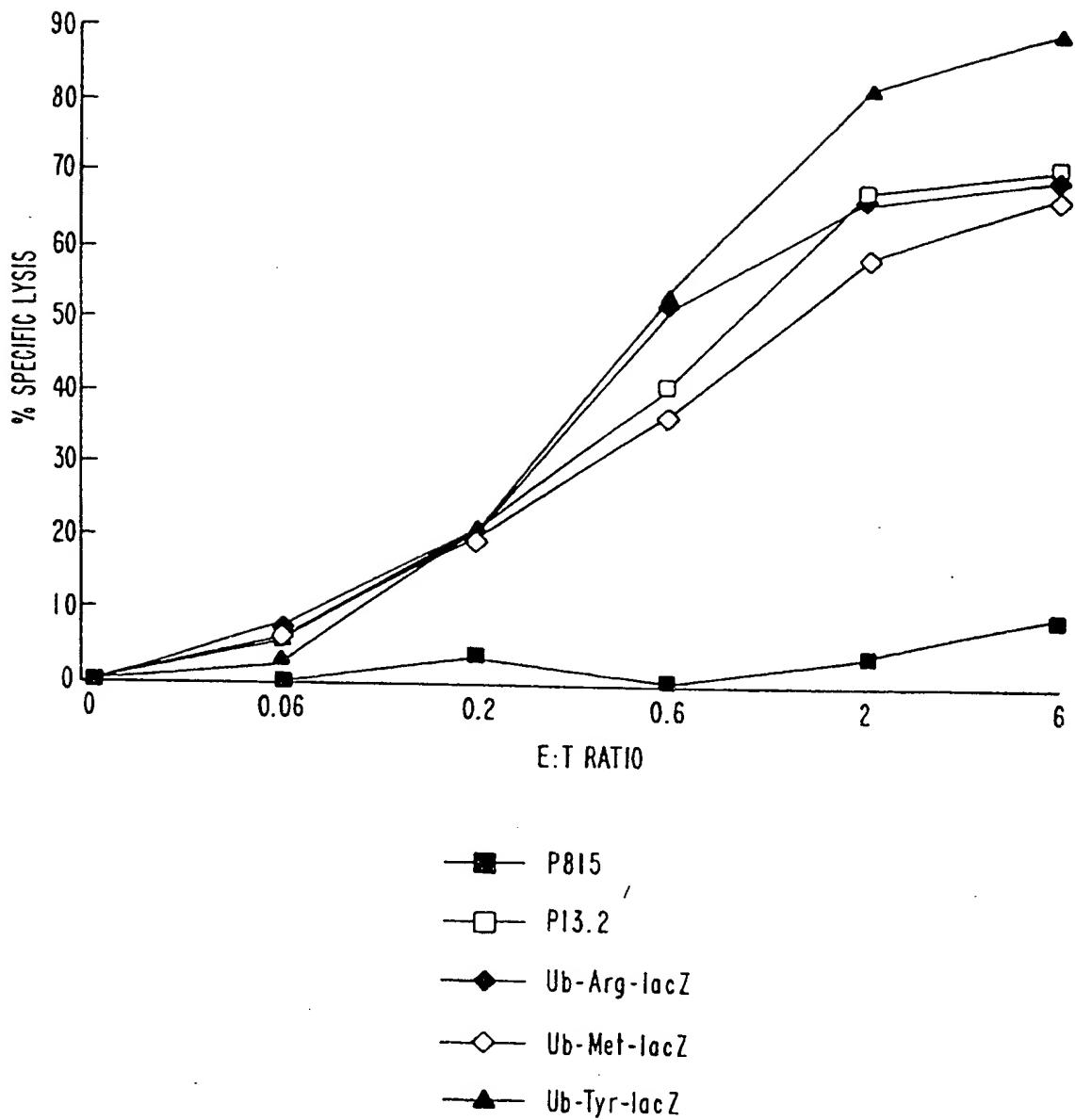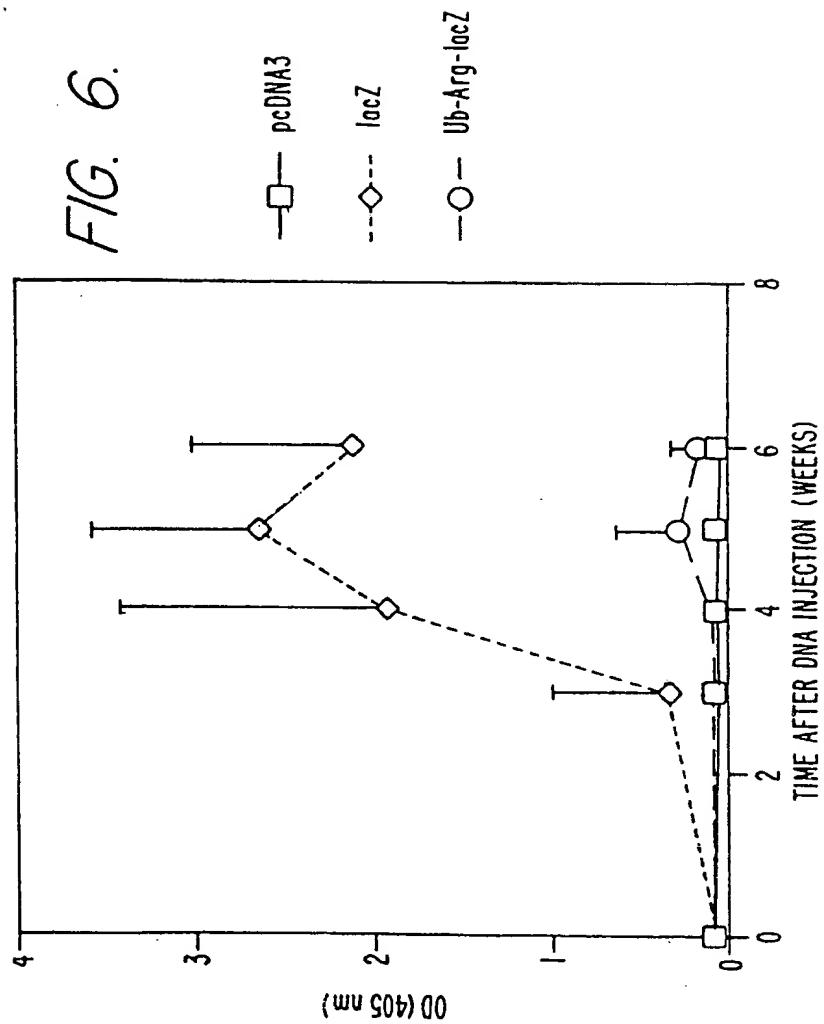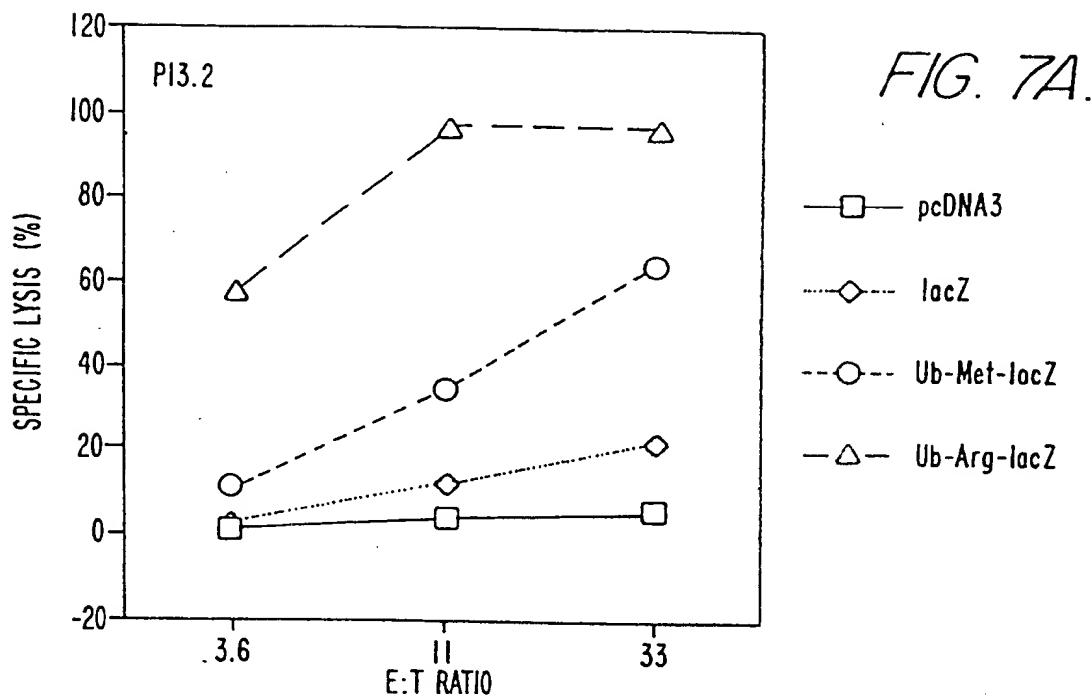
☑ CONTROL

☐ LLnL, 100 uM

▦ LACTACYSTIN, 100 uM

▩ CHLOROQUINE, 100 uM

◻ E64-D, 50 uM

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FIG. 5.

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FIG. 6.

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*FIG. 7A.*

P13.2

—□— pcDNA3

····◇···· lacZ

--○-- Ub-Met-lacZ

—△— Ub-Arg-lacZ

CELLULAR IMMUNE RESPONSE TO βgal IN BALB/c MICE IMMUNIZED WITH VARIOUS PLAMIDS.

FIG. 7B.

P815

—□— pcDNA3

····◇···· lacZ

--○-- Ub-Met-lacZ

—△— Ub-Arg-lacZ

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FIG. 8A.

Ub   ErbB-2/neu

|▨▨▨|—✕—|———————————————————|

|←————EXTRACELLULAR DOMAIN————→|←——CYTOPLASMIC DOMAIN——→|

FIG. 8B.

Ub   lacI   ErbB-2/neu

|▨▨▨|—✕—|▨|——————————————————|

|←————EXTRACELLULAR DOMAIN————→|←——CYTOPLASMIC DOMAIN——→|

FIG. 8C.

                                             AANDENYALAA

                   ErbB-2/neu

|———————————————————————————|▨|

|←——EXTRACELLULAR DOMAIN——→|←——CYTOPLASMIC DOMAIN——→|

FIG. 8D.

                                             ARINV

                   ErbB-2/neu

|———————————————————————————|▨|

|←——EXTRACELLULAR DOMAIN——→|←——CYTOPLASMIC DOMAIN——→|

FIG. 8E.

                                           ARINVARINV

                   ErbB-2/neu

|———————————————————————————|▨|

|←——EXTRACELLULAR DOMAIN——→|←——CYTOPLASMIC DOMAIN——→|

FIG. 8F.

                                         PEST

                   ErbB-2/neu

|———————————————————————————|▦▦|—ARINV

|←——EXTRACELLULAR DOMAIN——→|←——CYTOPLASMIC DOMAIN——→|

SUBSTITUTE SHEET (rule 26)
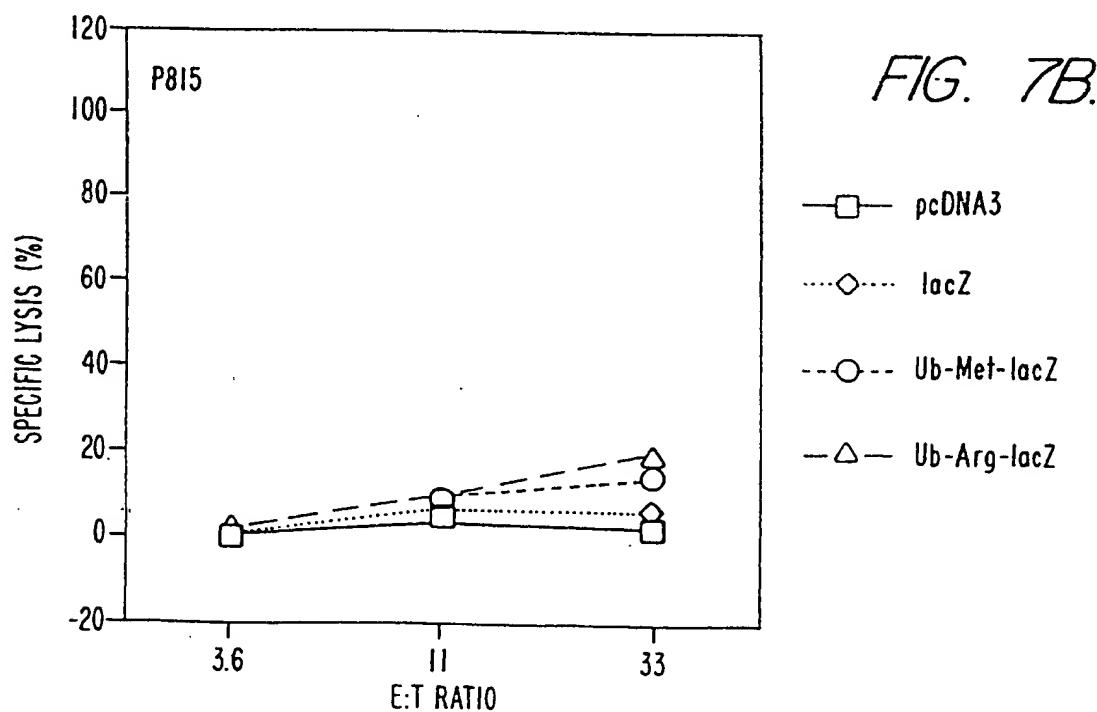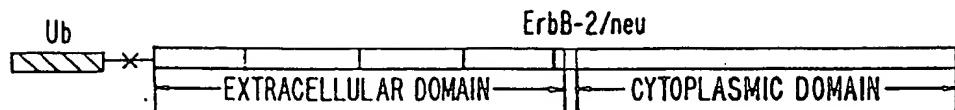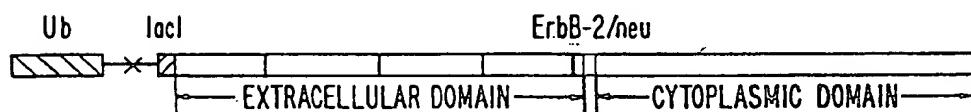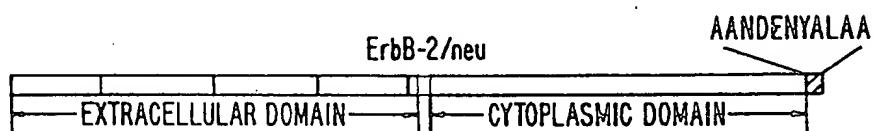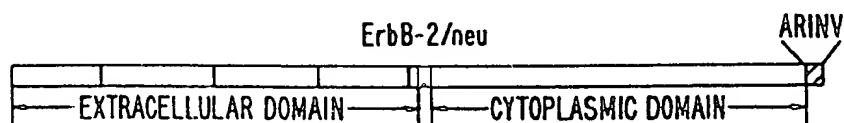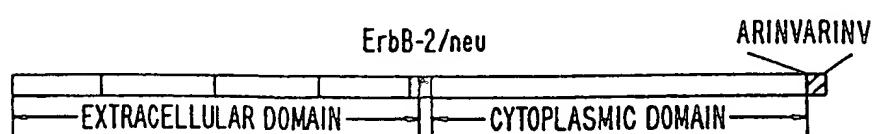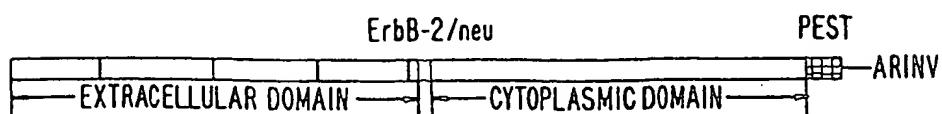
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/12   A61K48/00

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRANT ET AL: "RATE OF ANTIGEN DEGRADATON BY THE UBIQUITIN-PROTEASOME PATHWAY INFLUENCES MHC CLASS I PRESENTATION" THE JOURNAL OF IMMUNOLOGY, vol. 155, 1995, pages 3750-3758, XP002071199 cited in the application see the whole document ---	1-7
X	KEILER ET AL: "ROLE OF A PEPTIDE TAGGING SYSTEM IN DEGRADATION OF PROTEINS SYNTHESIZED FROM DAMAGED MESSENGER RNA" SCIENCE, vol. 271, 1996, pages 990-993, XP002071200 cited in the application see page 990 see abstract ---	7

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[X] Further documents are listed in the continuation of box C.	[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 July 1998	27/07/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	PEOPLES ET AL: "BREAST AND OVARIAN CANCER-SPECIFIC CYTOTOXIC T LYMPHOCYTES RECOGNIZE THE SAME HER2 / NEU-DERIVED PEPTIDE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 92, 1995, pages 432-436, XP002071201 cited in the application see page 432 see abstract	3
A	WO 97 11605 A (UNIV PITTSBURGH ;DANA FARBER CANCER INST INC (US)) 3 April 1997 see page 5, line 2 - page 6, line 7	3
P,X	WU ET AL: "DEOXYRIBONUCLEIC ACID VACCINES ENCODING ANTIGENS WITH RAPID PROTEASOME-DEPENDENT DEGRADATION ARE HIGHLY EFFICIENT INDUCERS OF CYTOLYTIC T LYMPHOCYTES" THE JOURNAL OF IMMUNOLOGY, vol. 159, 15 December 1997, pages 6037-6043, XP002071202 see the whole document	1-7

1.

INTERNATIONAL SEARCH REPORT

| Box I | Observations where certain claims were found unsearchabl (Continuation of Item 1 of first she t) |

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-6 are directed to a method of treatment of the human/ animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of Item 2 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Inter·   ·nal Application No
PCT/US 98/07290

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9711605 A	03-04-1997	AU 7251596 A	17-04-1997